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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12P 19/14, C12N 9/96 C11D 3/386, C12N 9/24	A1	(11) International Publication Number: WO 91/19807 (43) International Publication Date: 26 December 1991 (26.12.91)
(21) International Application Number: PCT/DK91/00158 (22) International Filing Date: 14 June 1991 (14.06.91) (30) Priority data: 1450/90 14 June 1990 (14.06.90) DK 91610023.3 27 March 1991 (27.03.91) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : ROSHOLM, Peter [DK/DK]; Nørre Søgade 15B, st/th, DK-1370 København K (DK). SI, Joan, Qi [DK/DK]; Taarbæk Strandvej 65A, DK-2930 Klampenborg (DK). GYALOKAY, Otto [DK/DK]; Sandkrogen 9, DK-2800 Lyngby (DK). WAGNER, Peter [DK/DK]; Krusågade 2, 3.th, DK-1719 København V (DK).		(74) Agent: NOVO NORDISK A/S; Patent Dept., Novo Allé, DK-2880 Bagsvaerd (DK). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i>
(54) Title: ACTIVATION OF POLYSACCHARIDE HYDROLASE		
(57) Abstract The effect of a polysaccharide hydrolase is increased by incorporation of a water-soluble polymer of one or more monomers selected from the group of vinyl pyrrolidone, vinyl alcohol, acrylamide and soluble acrylates into the reaction system in dissolved form.		

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ACTIVATION OF POLYSACCHARIDE HYDROLASE

TECHNICAL FIELD

This invention relates to a method of increasing the effect of a polysaccharide hydrolase.

5 BACKGROUND ART

Polysaccharide hydrolases are enzymes classified in group EC 3.2.1 and having a polysaccharide as substrate. Examples are endo-glucanases such as α -amylase, cellulase and pullulanase, which promote endo-hydrolysis of the glucans starch, cellulose and pullulan, respectively.

10 Many industrial uses of polysaccharide hydrolases are known. Thus, it is known to use pullulanase together with a saccharifying enzyme in the saccharification of liquefied starch for production of glucose (dextrose) or maltose (e.g. EP 63,909) or for the hydrolysis of pullulan into maltotriose. It is known to use cellulase e.g. for colour clarification of textiles (EP 220,016).

15 STATEMENT OF THE INVENTION

We have found that, surprisingly, addition of certain water-soluble polymers significantly increases the effect of polysaccharide hydrolases.

Accordingly, the invention provides a method of increasing the effect of a polysaccharide hydrolase, characterized by incorporation of a water-soluble
20 polymer of one or more monomers selected from the group of vinyl pyrrolidone, vinyl alcohol, acrylamide and soluble acrylates into the reaction system in dissolved form.

Another aspect of the invention provides an enzymatic process using a polysaccharide hydrolase, characterized by the presence of the above water-soluble polymer.

Finally, the invention provides a polysaccharide hydrolase preparation, characterized by comprising said water-soluble polymer.

PVP has been used as a binder in granulation of enzymes (e.g. protease, glucose isomerase) without any effect on enzyme activity being reported (e.g. US 4,572,897), PVP has also been reported to improve the storage stability of enzymes (e.g. peroxidase, protease) (e.g. EP 303,062, WO 90/00593). V. Jancsik, J.Mol.Catal., 6 (1), 41-50 (1979) reports inhibition of one kind of aldolase by PVP. F.E. Prado et al., J.Biol.Chem. 260 (8), 4952-7 (1985) reports activation of invertase by PVP. J.R.L. Walker et al., Phytochemistry (Oxford), 19 (3), 373-8 (1980) reports that o-diphenol oxidase (catecholase) is inhibited by PVP, whereas p-diphenol oxidase (laccase) is unaffected by PVP. PVP has also been used in the recovery of enzymes without any effect on enzyme activity being reported (EP 262,651, EP 214,531, US 4,144,130, US 4,016,039).

However, the prior art gives no guidance to the skilled person, enabling him to predict activation of polysaccharide hydrolase by PVP.

DETAILED DESCRIPTION OF THE INVENTION

Polysaccharide hydrolase

The invention is applicable to activation of a polysaccharide hydrolase, i.e. an enzyme in group EC 3.2.1 according to Enzyme Nomenclature having polysaccharide as substrate. It is particularly applicable to activation of an endoglucanase, i.e. an enzyme that effects endo-hydrolysis of a glucan (a polymer of glucose units), e.g. a cellulase, a pullulanase or an α -amylase.

Cellulase (EC 3.2.1.4) may be derived from *Humicola* (especially *H. lanuginosa*, US 4,435,307) or *Trichoderma* (e.g. *T. viride*, *T. reesei* or *T. koningii*) or may be prepared according to PCT/DK 90/00123. Pullulanase (EC 3.2.1.41) may be derived from *Bacillus*, particularly from *B. acidopullulyticus*, as described in EP

63,909. α -amylase (EC 3.2.1.1) may be derived from *Bacillus*, e.g. *B. amyloliquefaciens*, *B. licheniformis* or *B. stearothermophilus*.

The invention is also applicable to activation of inulinase (EC 3.2.1.7), derived e.g. from *Aspergillus*, especially *A. niger*.

5 Enzymatic process

The invention serves to increase the enzymatic effect in a process using a polysaccharide hydrolase.

An example is a process using cellulase for colour clarification of textiles, as described in EP 220,016. In colour clarification, the enzyme and PVP can
10 optionally be used together with a detergent.

Another example is saccharification of liquefied starch in the presence of pullulanase together with a saccharifying enzyme selected from the group of glucoamylase, β -amylase and maltogenic amylase. Another example is the use of pullulanase for hydrolysis of pullulan into maltotriose.

15 Water-soluble polymer

The water-soluble polymer used in the invention is a polymer of one or more of the monomers vinyl pyrrolidone, vinyl alcohol, vinyl carboxylate (e.g. C₁-C₄ carboxylate such as acetate), acrylamide and acrylate (e.g. an alkali metal acrylate such as sodium acrylate). The polymer may be a homo-polymer or a copolymer, e.g.
20 a block or random copolymer.

Examples of preferred polymers are PVP (polyvinyl pyrrolidone), PVA (polyvinyl alcohol), polyacrylate, and copolymers of vinyl pyrrolidone with acrylamide or vinyl alcohol.

PVP used in the invention preferably has an average molecular weight
25 of 5,000 - 1,000,000, particularly 5,000-50,000. The amount of PVP is preferably 0.005 - 5% in the reaction medium or 0.03-3 g PVP/kg dry solids at process conditions. In the enzyme preparation the amount of PVP is preferably 0.005 - 10%, particularly 0.1 - 5% (all percentages by weight). In the case of pullulanase, the amount of PVP is preferably $5 \cdot 10^{-4}$ - $5 \cdot 10^{-2}$ g PVP/PUN.

The amount of polymer is preferably 0.5-100 mg/l, e.g. 0.5-50 mg/l, in the reaction system or 0.005-10%, e.g. 0.1-5%, in the enzyme preparation.

PVA used in the invention is preferably a partially hydrolysed polyvinyl ester of a lower (e.g. C₁-C₄) carboxylic acid, especially polyvinyl acetate, which has a degree of hydrolysis above 75%, particularly above 90%, e.g. above 95%. The PVA preferably has a molecular weight of 5,000 - 200,000, e.g. 20,000 - 200,000, particularly 50,000 - 100,000.

The above-mentioned copolymers are preferably random copolymers having a ratio of vinyl pyrrolidone to vinyl alcohol or acrylamide in the range 1:10 to 10:1 (on molar basis).

Enzyme preparation

The enzyme preparation of the invention may be provided in the form of a stabilized liquid or non-dusting granulate, suited for storage and shipping, or it may be an aqueous solution, ready for use.

15 Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art.

Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar alcohol or a carbohydrate, e.g. sucrose in an amount of e.g. 20-80%, preferably above 50%. Lactic acid may also be used as a stabilizer, and the liquid may further contain a preservative such as sodium benzoate, typically 0.1-0.5%. A liquid pullulanase preparation according to the invention may further contain sodium sulphite or bisulphite corresponding to 0.1-2% as SO₂.

25 Depending on the intended use, the enzyme may be mixed with other active ingredients. Thus, pullulanase may be used as a mixed enzyme preparation containing pullulanase together with the above-mentioned saccharification enzyme. Cellulase together with PVP may be incorporated into a detergent composition, which may be liquid or granular.

Determination of cellulase activity (CEVU)

A substrate solution is prepared, containing 33.3 g/l CMC (Hercules 7 LFD) in 0.1 M Tris buffer at pH 9.0. The enzyme sample to be analyzed is dissolved in the same buffer. 10 ml substrate solution and 0.5 ml enzyme solution are mixed and transferred to a viscosimeter (e.g. Haake VT 181, NV sensor, 181 rpm) thermostated at 40°C. One Cellulase Viscosity Unit (CEVU) is defined as the amount of enzyme that reduces the viscosity to one half under these conditions.

Determination of pullulanase activity (PUN)

This unit is defined in EP 63,909.

10

EXAMPLES**EXAMPLE 1**Colour clarification of textile with *Humicola* cellulase and PVP

Black cotton swatches were treated by 3 repeated washings in a Terg-O-TometerTM each followed by a rinsing and drying process. Washing conditions were as follows:

	Liquid volume:	800 ml
	Agitation	100 movements/min.
	Washing time:	30 min.
	Washing temp:	40°C
20	pH	7.0
	Water hardness:	9 dH
	Textile:	100% cotton
	Amount of textile:	2 swatches/800 ml, each 10*15 cm
	Rinsing time:	30 min in running tap water
25	Drying:	line-drying, room temp (16 hours)

The detergent was a commercial US liquid detergent, used at a dosage of 2 g/l. Varying amounts of PVP (molecular weight 30,000) was added to the detergent.

Cellulase from *H. insolens* prepared according to US 4,435,307 was added to the detergent solution immediately before washing at a dosage of 0, 50 or 100 CEVU/l.

The surface of the swatches was analyzed by measuring reflected light. In this measurement, white light is projected onto the surface and the reflection/remission is detected at 16 wavelengths by means of an "Elrepho 2000" Datacolor Schweiz apparatus. The results of these detections are processed into Hunter coordinates of which the L-coordinate represents the grey scale value (100: black, 0: white).

The effect of the enzyme is removal of damaged cellulose fibers causing a grey/worn look, and by measuring the grey scale value a quantitative expression for the enzyme treatment is obtained. The results are expressed as delta L in relation to the surface treated without cellulase and PVP.

Detergent I:

	<u>0 CEVU/l</u>	<u>50 CEVU/l</u>	<u>100 CEVU/l</u>
0% PVP	0	1.14	1.38
0.1% PVP	0.25	1.49	1.8
0.5% PVP	0.17	1.64	1.84
1.0% PVP	0.11	1.82	1.87

It is seen that the colour clarifying effect of the cellulase is increased by addition of PVP.

EXAMPLE 2Colour clarification of textile with cellulase and PVA

This experiment was made in the same manner as in Example 1, but using a buffer system instead of detergent and PVA instead of PVP. The PVA had an average molecular weight of 72,000 and a degree of hydrolysis of 98%. The buffer system contained 5% (w/w) triethanol amine, 10% ethanol and 85% H₂O. A dosage of 2 g/l was used.

Results (expressed in Delta L as in Example 1):

	<u>0 CEVU/l</u>	<u>10 CEVU/l</u>	<u>25 CEVU/l</u>
0 mg/l PVA	0.0	0.21	0.53
10 100 mg/l PVA	-0.21	0.71	1.08

EXAMPLE 3Activation of cellulase with PVA/PVP copolymer

This experiment was made in the same manner as in Example 2, but using a random copolymer of vinyl alcohol and vinyl pyrrolidone instead of PVA.

15 Results:

	<u>0 CEVU/l</u>	<u>10 CEVU/l</u>	<u>25 CEVU/l</u>
0 mg/l PVA/PVP	0.0	0.21	0.53
100 mg/l PVA/PVP	0.15	0.98	1.08

EXAMPLE 4Activation of cellulase with PVP/acrylamide copolymer

This experiment was made in the same manner as in Example 2, but using a random copolymer of vinyl pyrrolidone and acrylamide instead of PVA.

5 Results:

	<u>0 CEVU/l</u>	<u>10 CEVU/l</u>	<u>25 CEVU/l</u>
0 mg/l copolymer	0.0	0.43	0.49
10 mg/l copolymer	0.06	0.44	0.99

EXAMPLE 5Activation of *Trichoderma* cellulase in CMC hydrolysis

10 Cellulase from *T. reesei* having an activity of 675 EGU/g (cellulase activity unit described in WO 91/07542) was tested with and without addition of PVP in hydrolysis of carboxymethyl cellulose (CMC). The amount of PVP was expressed as % by weight relative to the enzyme preparation.

A substrate solution was prepared, containing 34,0 g/l CMC (Hercules
15 7 LFD) in 0.1 M phosphate buffer at pH 6.0. The enzyme sample to be analyzed was dissolved in the same buffer. 5 ml substrate solution and 0.15 ml enzyme solution were mixed and transferred to a vibration viscosimeter (e.g. MIVI 3000 from Sofraser, France), thermostated at 40°C. The relative activity was calculated from the amount of enzyme that reduces the viscosity to one half under these conditions; the result
20 without addition of PVP was taken as 100. The amount of enzyme sample was 0.01-0.02 EGU/ml in the reaction mixture.

Results:

<u>% PVP</u>	<u>Relative activity</u>
0 (reference)	100
1%	103
3%	110

5 EXAMPLE 6

Activation of pullulanase in pullulan hydrolysis

A liquid pullulanase preparation was made according to EP 63,909. The preparation contained a pullulanase activity of 180 PUN/g and 50% sucrose.

Varying amounts of PVP (MW 15,000) were added to this preparation, and the apparent pullulanase activity was determined by the PUN method. PVP content is given as % by weight in the preparation. Results:

<u>% PVP</u>	<u>PUN/g</u>
0 (reference)	180
0.1	190
0.5	220
1.0	240

The results clearly show increasing activation up to 33% increase at 1.0% PVP.

EXAMPLE 7

Activation of pullulanase in saccharification

In this example, liquefied starch was saccharified with glucoamylase and pullulanase containing PVP. The experiments were run essentially as in EP 63,909.

The pullulanase samples of Example 6 were used and were dosed according to the apparent activity given in Example 6, i.e. correspondingly less of the preparations activated by PVP. The enzyme dosages were 0.18 AG/g of glucoamylase and 0.06 PUN/g of pullulanase. Saccharification conditions were 60°C, initial pH 4.3. Analyses by HPLC were made after 24, 48, 72 and 96 hours. The maximum % DP1 was as follows:

	<u>% PVP</u>	<u>max. % DP1</u>	<u>Max reached after hours</u>
	0	96.4	72
	0.1	96.4	72
10	1.0	96.4	72

Identical results were obtained, indicating that the same degree of activation is found in saccharification of hydrolyzed starch as in pullulan hydrolysis.

EXAMPLE 8

Activation of inulinase

15 Inulinase from *A. niger* was tested with and without addition of PVP in hydrolysis of inulin. The amount of PVP was expressed as % by weight relative to the enzyme preparation.

The conditions used for inulin hydrolysis were 50 g/l inulin (Inulin Sigma I 3754) in 0.025 citrate buffer at pH 4.70, 40°C, 30 minutes reaction time. The 20 concentration of reducing carbohydrates produced during the incubation was determined by means of the Somogyi Nelson reaction. The reference sample without PVP produced 3107 mol reducing carbohydrate (calculated as glucose) per g of enzyme preparation, and this was taken as 100%. Results:

	<u>% PVP in enzyme preparation</u>	<u>Relative inulin hydrolysis</u>
25	0 (reference)	100
	1%	107
	3%	111

CLAIMS

1. A method of increasing the effect of a polysaccharide hydrolase, characterized by incorporating a water-soluble polymer of one or more monomers selected from the group of vinyl pyrrolidone, vinyl alcohol, vinyl carboxylate, acrylamide and soluble acrylates into the reaction system in dissolved form.
2. A method according to Claim 1 of increasing the effect of an endo-glucanase, preferably a cellulase, α -amylase or pullulanase.
3. A method according to Claim 1 or 2, wherein the concentration of said polymer in the reaction system is 0.5-100 mg/l.
4. A method according to any of Claims 1 - 3, wherein said polymer is polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), polyacrylate or a copolymer of vinyl pyrrolidone with vinyl alcohol or acrylamide.
5. A method according to Claim 4, wherein said PVP has an average molecular weight of 5,000 - 1,000,000, preferably 5,000-50,000.
6. A method according to Claim 4, wherein said PVA is a partially hydrolyzed polyvinyl ester of a C_{1-4} carboxylic acid having a degree of hydrolysis above 75% and an average molecular weight of 5,000 - 200,000.
7. An enzymatic process using a polysaccharide hydrolase, characterized by the presence of a water-soluble polymer of one or more monomers selected from the group of vinyl pyrrolidone, vinyl alcohol, vinyl carboxylate, acrylamide and soluble acrylates in dissolved form.

8. A process according to Claim 7 using an endo-glucanase, preferably a cellulase, α -amylase or pullulanase.
9. A process according to Claim 8 using cellulase for colour clarification of cotton textile.
- 5 10. A process according to Claim 8 using pullulanase for the saccharification of liquefied starch together with a saccharifying enzyme selected from the group of glucoamylase, β -amylase and maltogenic amylase.
11. A polysaccharide hydrolase preparation, characterized by comprising a water-soluble polymer of one or more monomers selected from the group of vinyl
10 pyrrolidone, vinyl alcohol, acrylamide and soluble acrylates into the reaction system in dissolved form.
12. An endo-glucanase preparation according to Claim 11, preferably a cellulase, α -amylase or pullulanase preparation.
13. A mixed pullulanase preparation according to Claim 12, further
15 comprising a saccharifying enzyme selected from the group of glucoamylase, β -amylase and maltogenic amylase.
14. A preparation according to any of Claims 11 - 13, in the form of a non-dusting granulate or a stabilized liquid.
15. A detergent composition comprising cellulase according to Claim 12.
- 20 16. A preparation according to any of Claims 11-15, comprising 0.005 - 10%, preferably 0.1 - 5% of said polymer, by weight.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00158

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁵		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: C 12 P 19/14, C 12 N 9/96, C 11 D 3/386, C 12 N 9/24		
II. FIELDS SEARCHED		
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Classification System	Classification Symbols	
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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO, A1, 9000593 (NOVO NORDISK A/S) 25 January 1990, see specially the claims and page 16-17 --	1-2,4-8, 11-12, 14,16
X	US, A, 4176079 (RAYMOND E. GUERRY ET AL.) 27 November 1979, see the whole document --	1-4,6-8, 11-12, 14-16
X	Dialog Information Services, file 351, WPI, 1981-1991, Dialog Accession no. 7531906, Showa Denko KK: "Liq. or gel detergent compsn. contg. PVA coated enzymes - forming microcapsules and dispersing uniformly in the detergent; POLYVINYL ALCOHOL", JP 63105098, A, 880510, 8824 (Basic), see the whole abstract --	1-4,6-8, 11-12, 15
<p>[*] Special categories of cited documents: ¹⁰</p> <p>^{"A"} document defining the general state of the art which is not considered to be of particular relevance</p> <p>^{"E"} earlier document but published on or after the international filing date</p> <p>^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>^{"O"} document referring to an oral disclosure, use, exhibition or other means</p> <p>^{"P"} document published prior to the international filing date but later than the priority date claimed</p> <p>^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>^{"X"} document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>^{"Y"} document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>^{"&"} document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
16th September 1991	1991-09-19	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	Carl-Olof Gustafsson	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, volume 81, no. 7, 19 August 1974, (Columbus, Ohio, US), Irie, Akiko et al.: "Activation of alpha-amylase in urine ", see page 175, abstract 34870w, & Clin. Chim. Acta 1974, 51(3), 241- 245 --	1,2,4,7-8
A	US, A, 3860484 (JAMES JOSEPH O'MALLEY) 14 January 1975, see specially column 2, lines 30-36, 50-54 and example III --	1-8,11-14
A	EP, A2, 0220016 (NOVO INDUSTRI A/S) 29 April 1987, see the whole document --	1-3,8-9,11-15
A	Chemical Abstracts, volume 76, no. 14, 3 April 1972, (Columbus, Ohio, US), Ionescu-Stoian, P et al.: "Interaction of excipients with drugs. X. Influence of macromolecular nonionic agents on the enzymic activity of amylases ", see page 281, abstract 76402x, & Farmacia 1971, 19(9), 535- 542 --	1-2,4,7-8,11-12
A	Chemical Abstracts, volume 98, no. 7, 14 February 1983, (Columbus, Ohio, US), Reese, Elwyn T. et al.: "Protection of Trichoderma reesei cellulase from inactivation due to shaking ", see page 304, abstract 49392s, & Solution Behav. Surfactants 1980, 2(), 1487-1504 -- -----	1-3

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00158**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on **91-08-30**.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9000593	90-01-25	AU-D- 3978089 EP-A- 0311421 EP-A- 0351162	90-02-05 89-04-12 90-01-17
US-A- 4176079	79-11-27	NONE	
US-A- 3860484	75-01-14	NONE	
EP-A2- 0220016	87-04-29	JP-A- 62174298 US-A- 4738682	87-07-31 88-04-19